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HEALTH AND MEDICINE

ECRG4 regulates neutrophil recruitment and CD44 expression during the inflammatory response to injury

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The complex molecular microenvironment of the wound bed regulates the duration and degree of inflammation in the wound repair process, while its dysregulation leads to impaired healing. Understanding factors controlling this response provides therapeutic targets for inflammatory disease. Esophageal cancer–related gene 4 (ECRG4) is a candidate chemokine that is highly expressed on leukocytes. We used ECRG4 knockout (KO) mice to establish that the absence of ECRG4 leads to defective neutrophil recruitment with a delay in wound healing. An *in vitro* human promyelocyte model identified an ECRG4-mediated suppression of the hyaluronic acid receptor, CD44, a key receptor mediating inflammation resolution. In ECRG4 KO mouse leukocytes, there was an increase in CD44 expression, consistent with a model in which ECRG4 negatively regulates CD44 levels. Therefore, we propose a previously unidentified mechanism in which ECRG4 regulates early neutrophil recruitment and subsequent CD44-mediated resolution of inflammation.

INTRODUCTION

Cutaneous wound repair is a model of tightly controlled inflammation. Early inflammatory responses mediate hemostasis, recruitment of proreparative myeloid cells, and wound debridement to support the revascularization and proliferative phase of wound healing (1). Disruption of this inflammation response is associated with delayed wound closure, infection, and scarring (1–3). Therefore, understanding the mechanisms that regulate the degree and duration of the inflammatory response is critical for understanding and treating disease.

Injured tissues rapidly recruit leukocytes to clear pathogens and prepare the wound bed for tissue repair. This process involves some well-described chemokines, such as CXCL1 (4), CXCL2 (4), CXCL5 (5), and CXCL8 (6), and is further regulated by factors generated in the complex molecular environment of the wound. For example, proteases activate latent chemokines, such as chemoattractants generated in the complement cascade, and degradation of extracellular matrix components produces biologically active molecules, such as hyaluronan fragments (7, 8). These products mediate the recruitment and activation of leukocytes and stromal cells. Some receptors, such as the Toll-like receptor (TLR) family, amplify inflammation in response to damage-associated molecular patterns and pathogen-associated molecular patterns present in the wound, while others respond by tempering the inflammatory response. For example, activation of CD44 by hyaluronic acid (HA) fragments released from injured tissue modulates TLR signaling and can decrease inflammatory responses and prevent septic shock (9–11). CD44 is also important for resolving inflammation at sites of injury, a requirement for wound healing to progress to the proliferative phase. In injury models, such as cutaneous wounds (2), pneumonia (12), non-infectious lung injury (13), and myocardial infarction (14), loss of CD44 expression leads to increased and prolonged inflammation, exuberant leukocyte recruitment, failure to clear apoptotic neutrophils, and increased scarring and fibrosis (2, 7, 9, 12–15). Under-

standing the complex interaction of wound products and cellular receptors that govern the choreography of wound healing phase transitions is important for addressing poorly healing wounds and informs treatment of other inflammatory disorders.

Esophageal cancer–related gene 4 (ECRG4) is a membrane-tethered protein that is widely expressed in normal tissues and highly expressed on the surface of quiescent human granulocytes (16–18). ECRG4 was first described as an epigenetically regulated gene whose expression is suppressed in several cancer types (19–21), including esophageal cancer. Recent studies indicate that it may promote leukocyte recruitment, with its down-regulation in cancer contributing to escape from immunosurveillance (17, 22). ECRG4 physically associates with TLR4 (23), while proteolytic processing releases it from the cell surface (17, 24). Soluble ECRG4 binds various scavenger receptors, including LOX-1 (25), and regulates phosphorylation of the nuclear factor κ B (NF- κ B) transcription factor (16, 23). In murine tumor models, the ability of soluble ECRG4 to increase the recruitment of inflammatory cells is dependent on thrombin cleavage (17). In humans with severe burn injury, ECRG4 is shed from circulating neutrophils, while restoration of cell surface ECRG4 expression correlates with recovery (24). These observations suggest that ECRG4 has a role in regulating specific inflammatory responses, although its function in injury has not been determined in ECRG4 knockout (KO) mice. Here, we propose a previously unidentified mechanism in which the amplification of early neutrophil recruitment and CD44 expression in the wound bed can be regulated by proteolytic processing of the leukocyte chemokine ECRG4.

RESULTS

ECRG4 KO delays early wound closure and impairs initial neutrophil recruitment

To determine whether ECRG4 is a functional mediator of the wound healing response, ECRG4 KO and wild-type (WT) mice were subjected to splinted full-thickness excisional wounds. The wound area was measured daily to assess wound closure kinetics. ECRG4 KO mice had a notable, and statistically significant, delay in early wound closure compared to WT mice, which began at post-wound day 1

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(WT $22.5 \pm 2.4 \text{ mm}^2$ versus KO $30.2 \pm 0.7 \text{ mm}^2$) and persisted until day 9 (WT $6.8 \pm 4.7 \text{ mm}^2$ versus KO $15.6 \pm 4.2 \text{ mm}^2$) (Fig. 1, A and B). To visualize changes in the rate of wound closure, we plotted these data as the change in wound area over time (fig. S1A). This highlighted a slower initial rate of wound closure in ECRG4 KO mice after injury that subsequently recovered to the rate of WT mice by day 3. This represents the inflammatory phase of wound healing, suggesting a role for ECRG4 in regulating the initial inflammatory response. We examined the recruitment of Gr1⁺ granulocytes at the margin of 24-hour wounds and observed decreased Gr1⁺ staining in the ECRG4 KO mice compared to WT controls (Fig. 1C). To quantify the ECRG4-mediated defect in granulocyte recruitment, we used flow cytometry to immunophenotype single-cell suspensions of dissociated skin from

the margins of ECRG4 KO versus WT wounds (26). Flow cytometric analyses of CD45⁺ cells from the wound margins of post-injury day 1 animals demonstrated a ~14-fold increase in CD45⁺CD11b⁺Ly6G⁺ neutrophils in WT mice compared to uninjured skin, which was not seen in ECRG4 KO mice at the same time point (Fig. 1D, top). In contrast, there was no significant difference between the ECRG4 KO and WT wound neutrophil numbers at day 3 (Fig. 1D, bottom). This is the time at which the ECRG4 KO wound closure rate approaches that of WT mice (fig. S1A). There was also no difference in neutrophil populations between uninjured ECRG4 KO and WT skin. These findings are the first to use an ECRG4 KO mouse to establish the functional significance of ECRG4 in mediating neutrophil recruitment in the inflammatory phase of injury response.

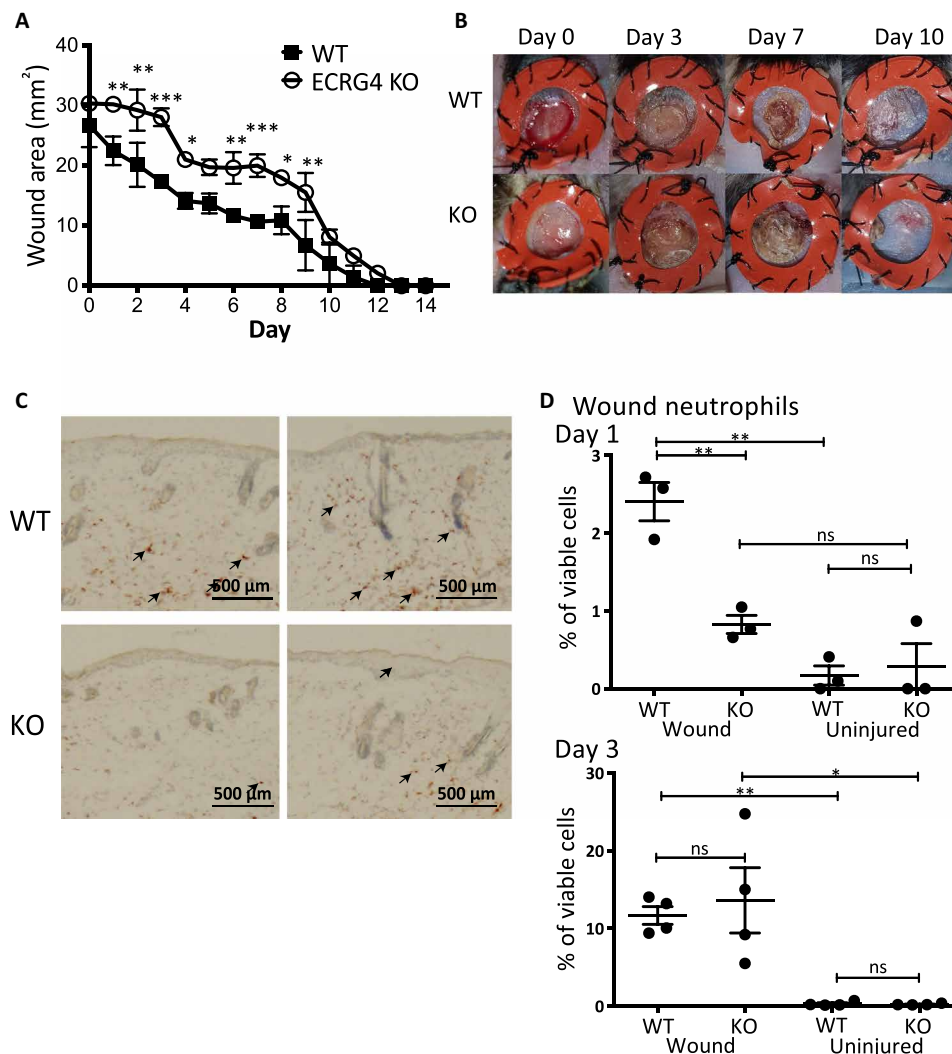


Fig. 1. ECRG4-deficient mice have delayed wound closure and neutrophil recruitment. To assess the role of ECRG4 in the cutaneous inflammatory response to injury, we examined wound healing as a model of tightly regulated inflammatory responses. (A) Splinted excisional wounds were created on the dorsal skin of ECRG4 KO and WT littermates. Wound area was measured daily [representative data from three separate experiments, $n = 6$ mice per group, two-way analysis of variance (ANOVA), $P < 0.0001$; Bonferroni posttest, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$]. (B) Representative images of wounds at various time points. Photo credit: Robert A. Dorschner, UCSD. (C) Excisional wounds in ECRG4 WT (top row) or KO mice (bottom row) were harvested for immunohistochemistry at 24 hours, and infiltration of Gr1⁺ granulocytes (arrows) was assessed. Two representative images from ECRG4 WT and KO wound margins are shown; scale bars, 500 μm ($n = 3$ per group). (D) Neutrophil recruitment to the full-thickness cutaneous wound was quantified using a protocol for preparing single-cell suspensions via partial enzymatic digestion followed by flow cytometry. Neutrophils were identified as CD45⁺CD11b⁺Ly6G⁺ cells in day 1 (top) and day 3 (bottom) excisional wounds ($n = 3$ to 4 mice per group; $**P < 0.01$ and $*P < 0.05$). ns, not significant.

ECRG4 regulates neutrophil recruitment to an aseptic injury

To define the role of ECRG4 in mediating leukocyte recruitment in the inflammatory phase of tissue response, we used subcutaneous implantation of polyvinyl alcohol (PVA) sponge, a well-characterized *in vivo* model of sterile inflammation and foreign body reaction (27–29). Because infiltrating leukocytes can be harvested from PVA sponges without the need for enzymatic preparation, the cellular infiltrate was directly immunophenotyped by flow cytometry. The 24-hour inflammatory infiltrate included subsets of CD11b⁺ leukocytes that could be differentiated by their expression of Ly6C, including CD11b⁺Ly6C^{int} neutrophils and CD11b⁺Ly6C^{high} inflammatory macrophages (Fig. 2, A to C), as demonstrated by Daley, Dunay, and Gutierrez (27, 30, 31) and confirmed in Fig. 2F. ECRG4 KO mice recruited half the number of total CD11b⁺Ly6C⁺ cells to the site of injury at 24 hours compared to their WT littermates (Fig. 2A). Further analysis of this population revealed a threefold decrease in the infiltration of CD11b⁺Ly6C^{int} neutrophils in the ECRG4 KO mice (Fig. 2B), whereas no significant changes in the recruitment of CD11b⁺Ly6C^{high} macrophages were observed (Fig. 2C). We further investigated the immunophenotype of the infiltrate at days 3 and 7 to identify any changes at these later time points. Similar to the wound (Fig. 1D), there was no difference in neutrophil recruitment between the WT

and ECRG4 KO mice at day 3 (fig. S1B) or day 7 (fig. S1C). Because the early wound environment is rich in thrombin and thrombin acts upon ECRG4 to release a biochemically active C-terminal peptide, CT16 (Fig. 2D) (16), that recruits leukocytes in a tumor model (17), we examined whether CT16 could increase leukocyte recruitment to a 24-hour injury. PVA sponges were loaded with 2 μ g of sterile synthetic CT16 peptide or vehicle before implantation. We found that CT16 specifically increased CD11b⁺Ly6C^{int} neutrophil recruitment by 35% at 24 hours (Fig. 2E). There was no difference in Ly6C^{low} macrophages or Ly6C^{high} inflammatory macrophages at this time point. These results demonstrate that ECRG4 is important for early neutrophil recruitment and support the role of ECRG4 as a novel protease-activated mediator of neutrophil recruitment in cutaneous injury.

Leukocyte production and their antimicrobial activity are not impaired in ECRG4 KO mice

We examined the leukocyte populations in blood and bone marrow to assess whether ECRG4 deficiency affected leukocyte production as a contributor to the decreased neutrophil numbers at the site of injury. Hemograms performed on the peripheral blood from uninjured mice demonstrated no differences between the circulating leukocyte populations of WT and ECRG4 KO mice (Fig. 3A). Similarly, flow cytometric

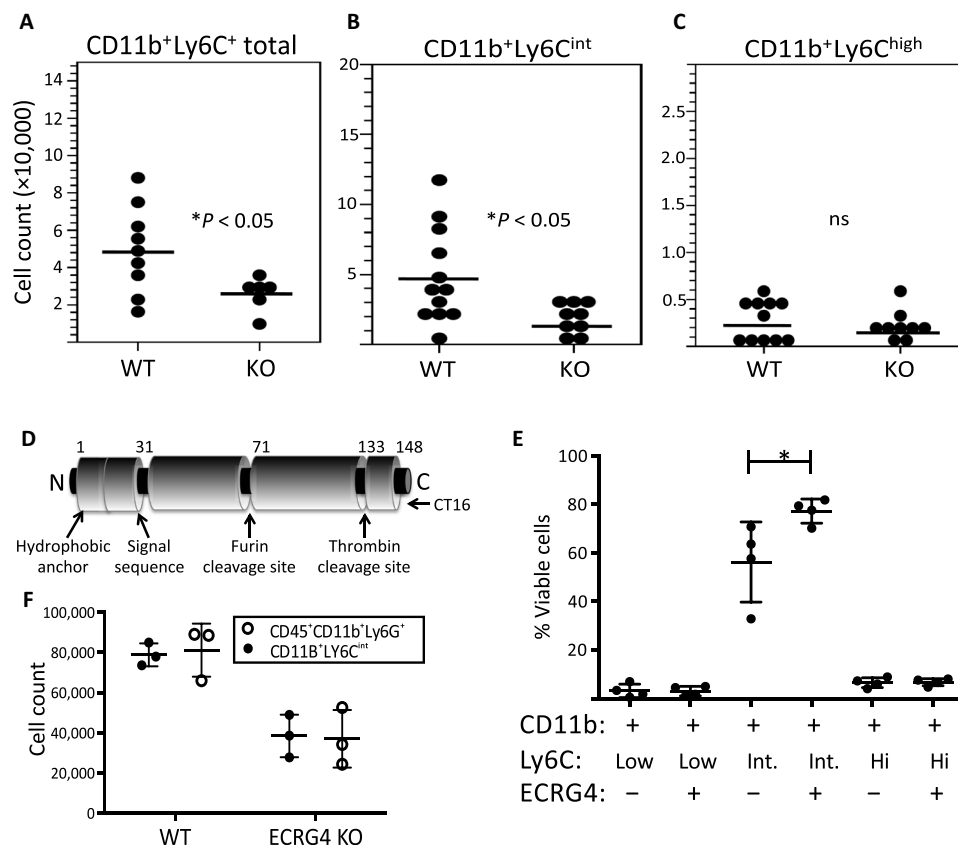


Fig. 2. ECRG4 deficiency impairs neutrophil response to an aseptic injury. The PVA aseptic injury model was used to model inflammatory responses at 24 hours. CD11b⁺ subpopulations were defined on the basis of their differential expression of Ly6C: CD11b⁺Ly6C^{low} patrolling macrophages, CD11b⁺Ly6C^{int} neutrophils, and CD11b⁺Ly6C^{high} inflammatory monocytes and macrophages. Quantification of (A) CD11b⁺Ly6C⁺ total, (B) CD11b⁺Ly6C^{int}, and (C) CD11b⁺Ly6C^{high} cells recruited at day 1 is shown ($n = 9$ to 12 mice per group; $*P < 0.05$). (D) The ECRG4 protein contains several domains, including a well-characterized thrombin cleavage domain, which releases the CT16 (ECRG4^{133–148}) peptide from the C terminus. (E) The addition of CT16 to the PVA sponge (2 μ g per sponge) increased recruitment of CD11b⁺Ly6C^{int} neutrophils at 24 hours, while CD11b⁺Ly6C^{low} and CD11b⁺Ly6C^{high} populations were not significantly different at this time point ($n = 4$; $*P < 0.05$). (F) Comparison of the CD11b⁺Ly6C gating strategy to the CD45⁺CD11b⁺Ly6C⁺ strategy for identifying neutrophils demonstrated equivalency in 24-hour PVA infiltrates and confirmed the decrease in ECRG4 KO neutrophil recruitment ($n = 3$).

analysis of bone marrow from uninjured mice demonstrated no difference in the composition or numbers of mature $CD11b^+Ly6C^+$ cell subsets (Fig. 3B). The composition of the ECRG4 KO mouse hematopoietic system was further analyzed by flow cytometry to assess populations of LSK stem cells and various progenitors of mature myeloid cells (Fig. 3, C and D). There were no differences in LSK hematopoietic stem cells (HSCs), long-term (LT)–HSCs, short-term (ST)–HSCs, multipotent progenitors (MPP), or LK myeloid progenitor cells (Fig. 3, E and F). Together, these data indicate that the lack of neutrophils seen at the site of injury in ECRG4 KO mice is not due to an impaired hematopoietic system, but a defect in local neutrophil recruitment. When we assessed the ability of ECRG4 KO mice

to kill the pathogen, *Staphylococcus aureus*, in blood, no functional defect in leukocyte antimicrobial activity was observed (Fig. 3G). There was no difference in the capacity for whole blood from either ECRG4 KO mice or their WT littermates to kill methicillin-resistant *S. aureus* (MRSA) as assessed ex vivo. This indicates that there is no defect in the antimicrobial function of ECRG4-deficient leukocytes.

Constitutive expression of ECRG4 reprograms leukocyte transcriptional activity in vitro

To assess the effect of ECRG4 on gene expression in an in vitro model of leukocytes, we performed an RNA sequencing (RNA-seq) analysis on HL60 human promyelocyte cells engineered to constitutively

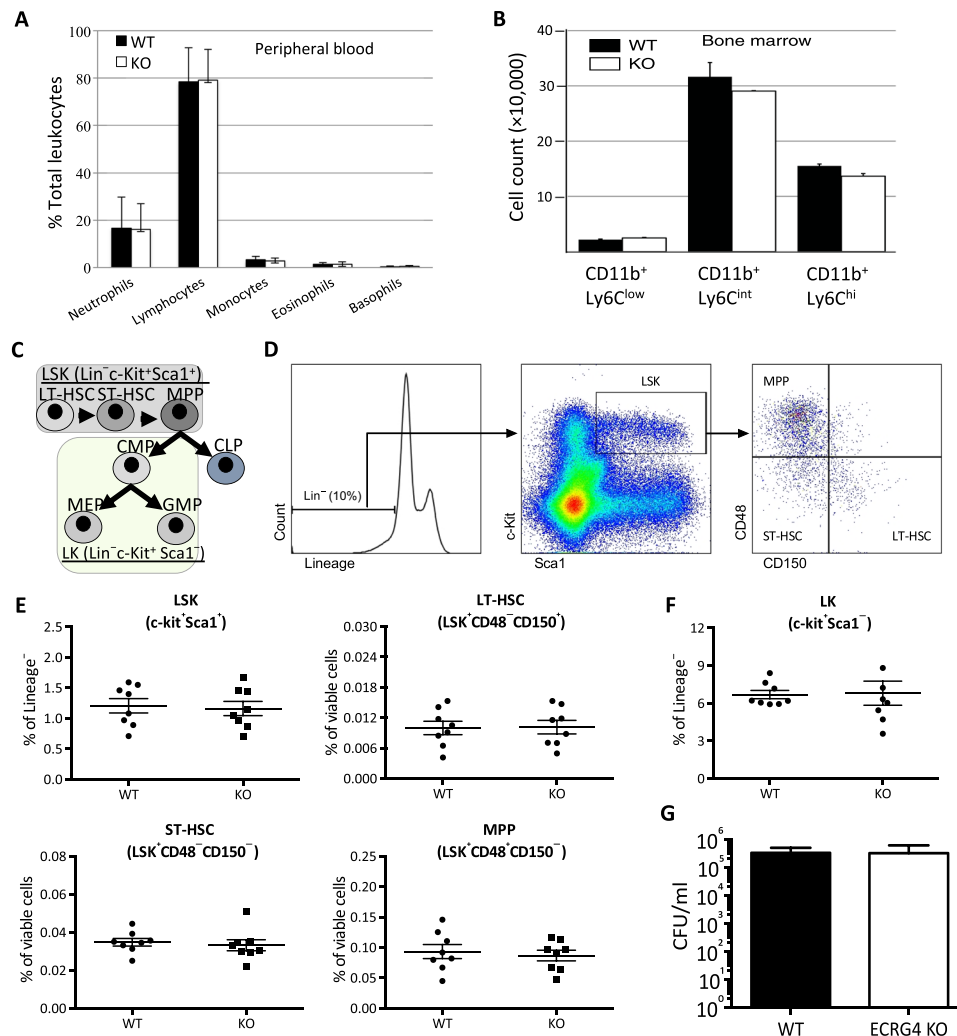


Fig. 3. ECRG4 deficiency does not affect myelopoiesis or leukocyte antimicrobial function. (A) Leukocyte populations in uninjured ECRG4 KO and WT mice were assessed for underlying deficits. Hemograms of blood showed no significant difference in populations between uninjured ECRG4 KO (white bars) and WT (black bars) mice ($n=8$ mice per group). (B) Flow cytometric analysis of bone marrow from uninjured ECRG4 KO (white bars) and WT (black bars) mice showed no significant difference between populations of $CD11b^+Ly6C^{low}$, $CD11b^+Ly6C^{int}$, and $CD11b^+Ly6C^{high}$ cells ($n=9$). HSC compositions were compared between the WT and ECRG4 KO bone marrow using flow cytometry. (C) Schematic of the lineage distinctions for HSCs and hematopoietic progenitor cells evaluated. (D) Lin^- cells from healthy ECRG4 KO or WT bone marrow (left) were gating on the $c-Kit^+Sca1^+$ LSK population (middle), which was then differentiated by CD48 and CD150 expression (right) to identify long-term (LT) HSC, short term (ST) HSC, and multipotent progenitor (MPP) populations. (E) There was no detectable difference in LSK HSCs ($Lin^-c-Kit^+Sca1^+$), including no difference in LT HSC, ST HSC, or MPP subpopulations. (F) In addition, there was no difference in numbers of LK HPCs ($Lin^-c-Kit^+Sca1^-$), which include common myeloid progenitor (CMP), megakaryocyte/erythrocyte progenitor (MEP), and granulocyte/macrophage progenitor (GMP) populations that give rise to all nonlymphoid myeloid cells ($n=8$ mice per group). (G) The antimicrobial function of ECRG4 KO leukocytes was compared to WT mice in whole blood for their ability to kill MRSA. USA300 LAC [10^6 colony-forming units (CFU)/ml] was inoculated into fresh whole blood and incubated at 37°C for 3 hours, and the remaining colonies were enumerated, revealing no difference in antimicrobial function ($n=3$ mice per group).

express ECRG4. In a comparison with vector control HL60 cells, 1638 genes were up-regulated and 1632 were down-regulated in cells constitutively expressing ECRG4 (data file S1). Both Gene Ontology analysis of biochemical processes and a KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway visualization analysis demonstrated a number of biochemical pathways affected by the constitutive expression of ECRG4 that are important for inflammation and injury response (Fig. 4A and table S1).

The pathway most affected by the expression of ECRG4 in the KEGG analysis was the category of proteoglycans in cancer (Fig. 4A), which included changes in the expression of the HA receptor, CD44, and its signaling pathways (Fig. 4B and fig. S2). We validated a number of targets with potential relevance to the ECRG4-dependent inflammatory response defect in ECRG4 KO mice by flow cytometry (fig. S3), and cell surface CD44 was the most markedly altered. Given the deficit in neutrophil recruitment and wound healing seen in the ECRG4 KO mouse and the importance of CD44 in regulating leukocyte migration, inflammation, and TLR signaling (2, 9–11, 13, 14, 32), we hypothesized that ECRG4 may regulate the expression of CD44. Using these HL60 cells *in vitro*, we verified the RNA-seq data with quantitative polymerase chain reaction (PCR) to measure mRNA and flow cytometry to measure surface protein (Fig. 4, C and D). Delivery of ECRG4 leads to a decrease of cell surface CD44 protein in HL60 cells (Fig. 4C) and CD44 gene expression (Fig. 4D) compared to control cells. On the basis of the observation that shed ECRG4 peptide increases neutrophil recruitment *in vivo* (Fig. 2E), we determined whether soluble ECRG4 affected CD44 expression. Incubation of naïve HL60 cells with the conditioned media of ECRG4-expressing cells, but not control cells, decreased CD44 surface protein expression (Fig. 4E). These results suggest that soluble ECRG4 can negatively regulate CD44 expression *in vitro*.

CD44 expression is increased on ECRG4 KO mouse neutrophils

On the basis of the observation that increased expression of ECRG4 down-regulated CD44 on myelocytes *in vitro*, we sought to determine whether the loss of ECRG4 in KO mice affected CD44 on leukocytes *in vivo*. Using a flow cytometric analysis of uninjured mice, ECRG4 KO mice demonstrated a 57% increase in the expression of CD44 on blood neutrophils compared to WT controls (Fig. 5, A and C), with no difference in the overall number of CD44⁺ neutrophils (Fig. 5B). In bone marrow from uninjured mice, we observed an increase in CD44 surface expression on ECRG4 KO neutrophils (22.7% increase; Fig. 5, D and F) compared to WT mice, with no significant difference in the number of CD45⁺ cells or CD11b⁺Ly6G⁺ neutrophils (Fig. 5E and fig. S4). This increased CD44 expression on ECRG4 KO leukocytes, particularly neutrophils, correlates inversely with the loss of CD44 seen on myeloid cells overexpressing ECRG4 *in vitro* and supports a role for ECRG4 in the negative regulation of CD44 expression on neutrophils.

ECRG4 and CD44 expression are inversely correlated in the mouse wound

On the basis of the *in vitro* and *in vivo* data demonstrating that ECRG4 negatively regulates CD44 expression on leukocytes, we evaluated the expression of these genes over the entire course of wound healing in mouse skin. Previous studies by our group demonstrated that ECRG4 expression remains low for the first 3 days after cutaneous injury and then increases with a peak of gene expression at day 7,

when the wound is transitioning from the proliferative to the remodeling phases of repair (33). We reanalyzed the same gene expression database of cutaneous wound repair (34) and found that, as predicted, CD44 gene expression rapidly increased after injury and peaked at 24 hours before gradually returning to its baseline expression (Fig. 5G). As reported by Shaterian *et al.*, ECRG4 expression remained low until day 3, when it increased until day 7, and then gradually decreased toward baseline (Fig. 5H). To compare these changes, expression was normalized to their relative expression at time 0 before injury and assessed together (Fig. 5I). The inverse relationship between ECRG4 and CD44 expression in the murine cutaneous wound supports a model wherein ECRG4 negatively regulates CD44 to mediate the inflammatory response to cutaneous injury.

DISCUSSION

This study demonstrates a role for leukocyte ECRG4 as a factor that amplifies neutrophil recruitment to site of injury and regulates their expression of CD44. Coordination of inflammation is an essential component of injury response and effective wound healing. The recruitment of neutrophils is a hallmark of the early wound response during hemostasis and the initial inflammatory phase, which prepares the wound bed for subsequent reparative leukocytes to mediate revascularization, reepithelialization, and tissue repair. The regulation of CD44 by ECRG4 in this process is consistent with the role of CD44 in resolving inflammatory processes, including limiting neutrophil accumulation at sites of injury (2, 14), tempering TLR-mediated inflammatory responses (9–11), and enhancing phagocytosis (15) and efferocytosis (13), all of which are central to coordinated wound healing.

This study uses a novel genetic KO mouse model to identify ECRG4 as a previously unrecognized regulator of inflammation in cutaneous injury. Analysis of these wounds demonstrated defective recruitment of neutrophils in the ECRG4 KO wound at 24 hours after injury, which was confirmed in an aseptic injury model. We evaluated whether a thrombin-mediated ECRG4 peptide could contribute to this effect, because it has been shown to enhance leukocyte recruitment in tumor models. The CT16 peptide of ECRG4 was able to increase early *in vivo* neutrophil recruitment to an aseptic injury, demonstrating that an ECRG4 peptide generated in the thrombin-rich inflammatory microenvironment of the wound bed amplifies neutrophil responses. These data define ECRG4 as a regulator of the inflammatory response to injury, including early neutrophil recruitment and wound healing.

To further define the effects of ECRG4 seen in leukocytes *in vivo*, we used HL60 cells as an *in vitro* myeloid cell model. We identified the alteration of a number of key inflammatory processes in cells constitutively expressing ECRG4, including innate immune signaling (TLR signaling, NF- κ B cascade, and cytokine signaling), apoptosis, and cell cycle. Among these, CD44 expression was completely abrogated at the transcriptional and protein expression levels in the HL60 cells expressing ECRG4. This effect is mediated, at least in part, by a soluble factor from the ECRG4-expressing cells, as their conditioned media decreased CD44 expression on naïve cells. These results indicate that ECRG4 can function in an autocrine or paracrine fashion to affect transcriptional programming of cells in the local inflammatory milieu. While it has been shown that various ECRG4 peptides are released into the media of cells constitutively expressing

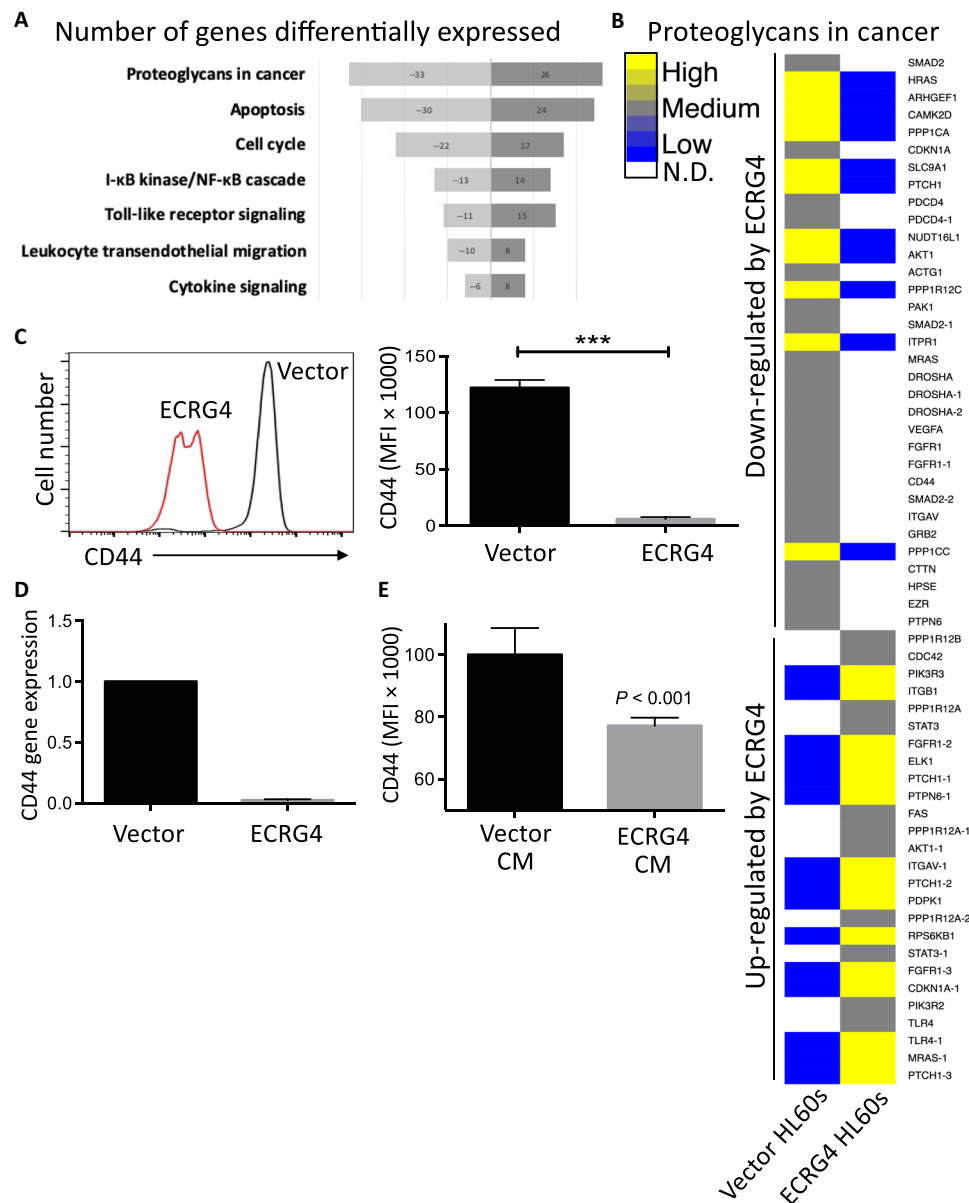


Fig. 4. Constitutive expression of ECRG4 in HL60 promyelocytes reprograms inflammatory response pathways. RNA-seq was performed on HL60 promyelocytes constitutively expressing ECRG4. (A) The top differentially expressed pathways, as determined by KEGG pathway visualization, included pathways central to inflammatory responses. (B) The top differentially expressed category was proteoglycan signaling, with the CD44 pathway having the most genes altered, as demonstrated in the heat map (N.D., not detected). (C) CD44 cell surface protein was evaluated on these cells via flow cytometry, gating on green fluorescent protein–positive (GFP⁺) cells and measuring CD44 mean fluorescence intensity (data are representative of five separate experiments with three replicates each; ***P < 0.001). (D) Confirmation of CD44 gene expression in the H60 cells constitutively expressing ECRG4 demonstrated loss of CD44 transcription via quantitative PCR (data are representative of five separate experiments with three replicates each). The ability of a soluble factor from the cells constitutively expressing ECRG4 to suppress CD44 was demonstrated by the addition of conditioned media (CM) to naïve parental HL60 cells. (E) Decreased CD44 cell surface protein expression was determined by flow cytometry (data are representative of five separate experiments with three replicates each). MFI, mean fluorescence intensity.

ECRG4 (16), the precise mechanism for its suppression of CD44 expression is unknown. We show that the capacity for ECRG4 to regulate CD44 expression *in vivo* based on the increased CD44 expression observed on neutrophils from ECRG4 KO mice compared to WT mice and the inverse expression of these genes in the murine wound. These changes were due to an increase in CD44 expression per cell and not a change in the overall number of CD44⁺

cells in blood or bone marrow, supporting the role of ECRG4 as a negative regulator of CD44.

These findings define ECRG4 as a mediator of CD44 expression and support a model where loss of ECRG4 at the site of injury increases CD44 expression, suggesting a novel mechanism for the regulation of the inflammatory response in cutaneous injury. In these studies, we demonstrate a role for ECRG4 in both cutaneous

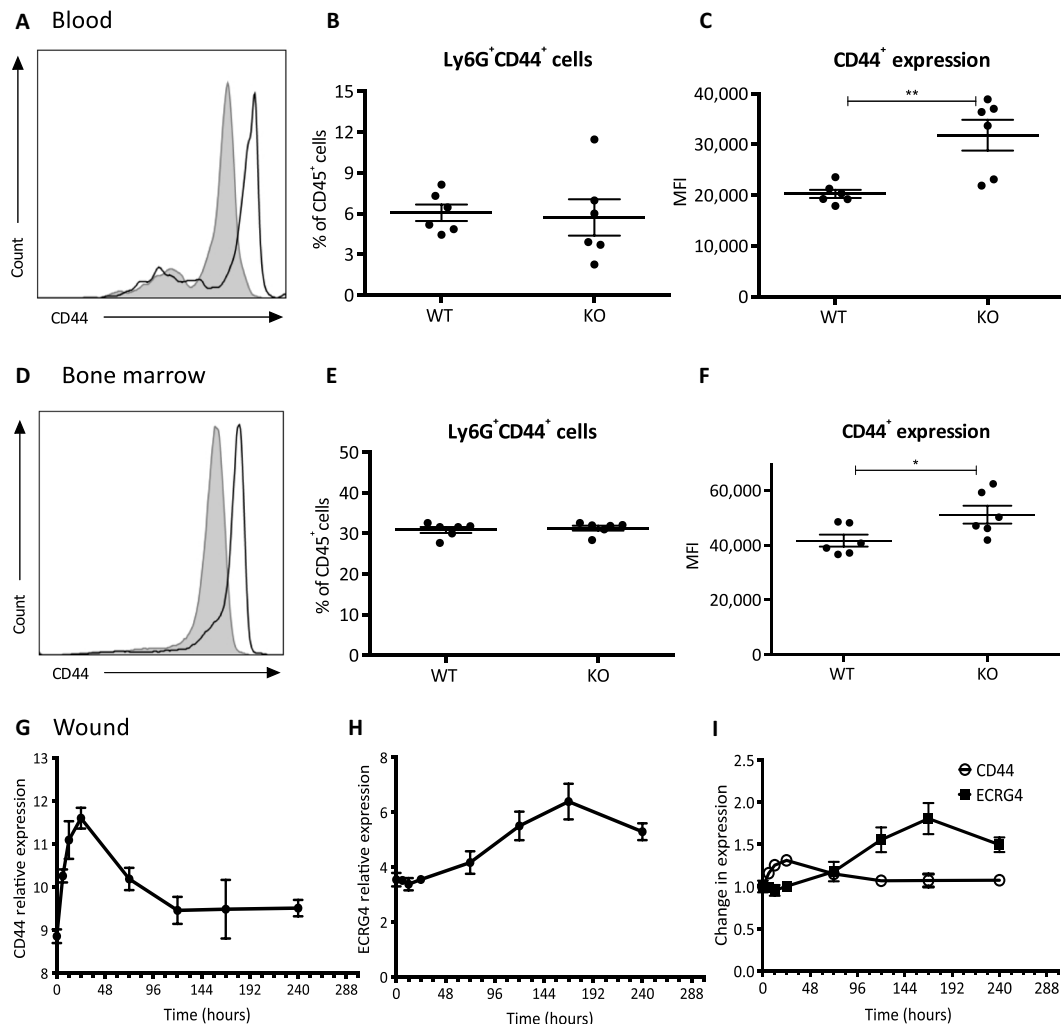


Fig. 5. Loss of ECRG4 increases CD44 expression in vivo. Flow cytometric analysis of blood (A to C) and bone marrow (D to F) from healthy ECRG4 KO mice and littermate controls assessed CD44 expression. (A and D) Representative histograms of CD44 expression on ECRG4 KO (unshaded plot) and WT (shaded plot) neutrophils. (B and E) Middle: Number of CD44⁺ cells within the CD45⁺CD11b⁺Ly6G⁺ neutrophil population, which were not significantly different between the ECRG4 KO and WT mice ($n = 6$ mice per group, representative of two independent experiments). (C and F) Right: CD44 MFI on CD45⁺CD11b⁺Ly6G⁺ neutrophils, which show a significant increase in the amount of CD44 expressed per cell in the ECRG4 KO mice compared to their WT controls [$**P < 0.01$ for (C) and $*P < 0.05$ for (D); $n = 6$ mice per group, representative of two independent experiments]. The expression of ECRG4 and CD44 was correlated in vivo in a wound time course. Gene expression was analyzed from an Affymetrix microarray performed on murine cutaneous wounds at multiple times through the wound healing process [Gene Expression Omnibus accession GSE23006; (34)]. Relative expression of CD44 (G) and ECRG4 (H) was plotted against time. (I) To visualize changes in expression of CD44 and ECRG4 together, the data were normalized to their expression at time 0 (uninjured skin). $n = 3$ mice for each of the eight time points.

wounds and the subcutaneous PVA implant, which represent two distinct models of inflammation. This also appears to be a previously unrecognized regulatory pathway of inflammation, as CD44 itself is known to play an important role in regulating the inflammatory response to a variety of injuries, including cutaneous wounds (2), pneumonia (12), noninfectious lung injury (13), and myocardial infarction (14). Neutrophil recruitment and TLR responses are central to the cutaneous host defense against infection. Several investigators have found that activation of CD44 by fragments of HA released from injured tissue modulates TLR signaling, decreases inflammatory responses, and prevents excessive systemic inflammation, such as septic shock (9–11). Others have shown that decreased CD44 enhances neutrophil accumulation at the site of injury (2, 14). Together,

these data infer that an ECRG4-CD44 pathway may be important for host defense.

Our findings demonstrate that ECRG4 mediates the early inflammatory response and regulates CD44 expression. In addition to this ability, previous studies have shown that cell surface ECRG4 can be processed by proteases, like thrombin, at the site of inflammation or tumor to release peptides with proinflammatory activity. While intact ECRG4 associates with TLR4 (16, 23), its various peptide fragments increase phosphorylation of p65 (16, 17), bind scavenger receptors, and activate NF- κ B in a MyD88-dependent manner in cultured cells (25). Despite these reports, the function of ECRG4 in regulating inflammation was previously unknown, in vivo.

On the basis of our current findings, we propose a model (Fig. 6) where ECRG4 acts as a sentinel and maintains homeostasis on

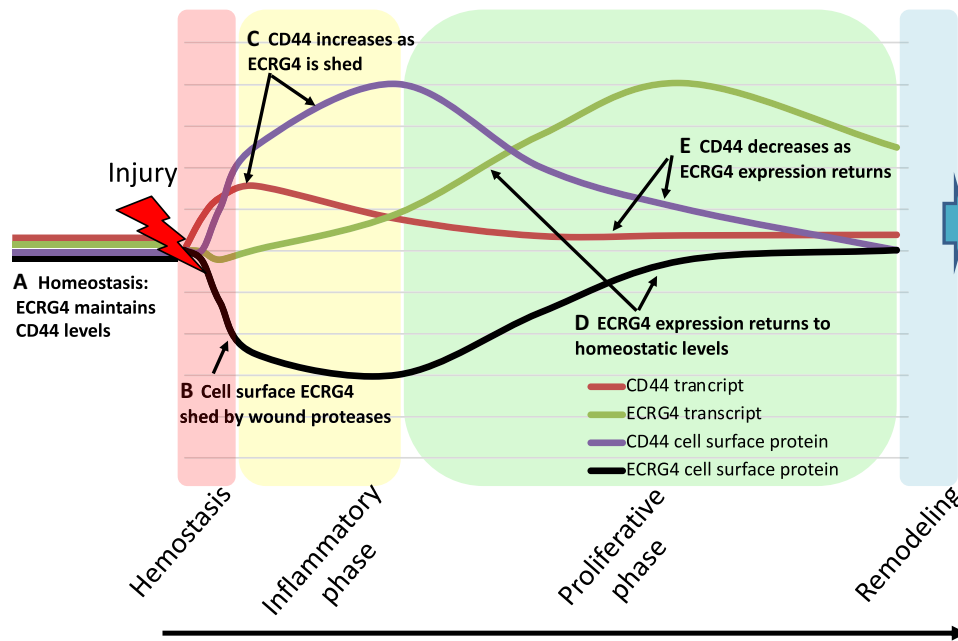


Fig. 6. Model: ECRG4-CD44 dynamics on leukocytes mediate the kinetics of wound inflammation. (A) On quiescent leukocytes, ECRG4 expression maintains CD44 at homeostatic levels. Following injury, leukocytes present in the wound are exposed to proteases during the hemostasis and early inflammatory phases. These proteases cleave leukocyte cell surface ECRG4 to release active peptides into the wound milieu, which enhance the early local inflammatory response. (B) Black tracing represents decreasing cell surface ECRG4 as it is cleaved by wound proteases to release peptides. During this phase, ECRG4 transcription remains low (green tracing), so ECRG4 protein is not replenished on the cell surface. (C) This loss of ECRG4 removes its inhibition of CD44 expression (red tracing), which results in an increase in cell surface CD44 receptor (purple tracing). Increased CD44 functions to resolve the inflammatory phase; it is activated by HA fragments in the wound to decrease proinflammatory signaling. As the wound transitions to the proliferative phase, ECRG4 expression resumes (D) and mediates a decrease in CD44 expression (E), returning both ECRG4 and CD44 cell surface protein to homeostatic levels as wound closure completes and the wound transitions to the remodeling phase. Gene expression data from Fig. 5 (G to I) [Gene Expression Omnibus accession GSE23006; (34)] are shown. Protein expression inferred from published reports (14, 35).

quiescent leukocytes, including restraining expression of CD44. In this model, ECRG4 expression levels determine the amount of CD44 on the leukocyte surface (Figs. 5I and 6A) to precondition its responsiveness to proinflammatory signals, such as TLR agonists. Higher levels of ECRG4 expression would result in decreased CD44 and thus less anti-inflammatory signaling, such as TLR signal suppression. After injury, rapid processing of cell surface ECRG4 protein in the protease-rich inflammatory milieu releases ECRG4 peptides that activate NF- κ B signaling and amplify local inflammatory responses and neutrophil recruitment (Fig. 2E). This proteolytic processing depletes cell surface ECRG4 (Fig. 6B), while ECRG4 transcription remains low immediately after injury, preventing its replacement (Figs. 5H and 6) (33–35). Loss of ECRG4 releases its inhibition of CD44. This leads to subsequent increases in CD44 expression at the site of injury (Figs. 6C and 5, G and I) (14, 34), which can temper proinflammatory responses (9–11) and contribute to the resolution of inflammation (2, 12–14). Return of ECRG4 expression at the end of the inflammatory phase (Figs. 6D and 5, H and I) decreases CD44 expression (Figs. 6E and 5, G and I) and restores homeostatic expression levels. This model is further supported by studies of human burn patients, where it has been observed that cell surface ECRG4 on circulating leukocytes diminishes after injury but returns to house-keeping levels as patients recover (24). These findings support a role for ECRG4 as a sentinel factor, which can both precondition the responsiveness of leukocytes through its regulation of CD44 expression and respond to environmental cues to mediate the kinetics of the cutaneous inflammatory response.

While deficient neutrophil mobilization has been shown to inhibit wound healing (36, 37), which we observed in the ECRG4 KO mouse wound, many studies have demonstrated that prolonged neutrophil recruitment also results in delayed wound closure (38, 39). Earlier studies examined the effect of adenovirus-driven ECRG4 overexpression in cutaneous excisional wounds and observed delayed wound healing, possibly through decreased migration of fibroblasts (33). On the basis of our current studies, we hypothesize that ECRG4 overexpression in the wound bed decreases CD44 expression, which has been shown to decrease fibroblast migration (2). This is supported by the wound closure (Fig. 1A) and healing rate (fig. S1A) plots. Initially, rapid wound closure occurs through contraction and migration of keratinocytes over residual basement membrane at the wound margin. The rate then slows, as further reepithelialization requires keratinocyte proliferation and replacement of the basement membrane by fibroblasts. In our model, ECRG4 KO mice have increased CD44 expression, which may contribute to the equalization of the healing rate seen in the KO mouse after an initial delay that occurs in the first 3 days after injury. The potential role of ECRG4 in regulating the function of stromal cells is outside the scope of this study but will be an important area for further investigation. Our current results also suggest that an ECRG4-mediated decrease in CD44 expression could support excessive neutrophil recruitment and prolonged inflammation (2, 14, 32), which also contribute to delayed wound closure. Neutrophils are essential for the onset of inflammation, but the termination of neutrophil influx is equally critical for

timely resolution of inflammation and prompt restoration of tissue homeostasis (40).

In our study, we demonstrated that ECRG4 is a negative regulator of CD44 expression and a mediator of early inflammatory responses. It is interesting to note that the gene encoding ECRG4 is epigenetically regulated. Its expression and transcriptional responsiveness are down-regulated by hypermethylation of its promoter (41), accounting for its diminished expression in multiple human cancers (19–21). Many of these same cancers are marked by increased expression of CD44 (42), which is implicated in their proliferation and metastasis and correlates with our findings that ECRG4 is a negative regulator of CD44. On the basis of our experimental observations demonstrating a role for ECRG4 in recruiting neutrophils to the site of injury and regulating CD44 expression levels, it is interesting to speculate that the epigenetic inhibition of ECRG4 expression may alter CD44 expression to precondition the inflammatory response, thus driving phenotypic differences in individuals based on environmental exposures and aging, which affect promoter methylation.

Here, we report that ECRG4 modulates CD44 expression and mediates the early neutrophil response to injury, with impaired wound repair in its absence. The precise timing of inflammation and its resolution in the wound healing response is essential to proper wound closure, with unresolved inflammation driving most of the chronic wounds. Our current study demonstrates that ECRG4 is a factor that helps orchestrate the proper timing of the inflammatory response: First, it facilitates and amplifies early inflammation but then subsequently supports the expression of factors that are essential to injury resolution. A better understanding of its contribution to inflammation preconditioning, susceptibility, and resilience will help identify new and sorely needed diagnostic and therapeutic strategies to treat cutaneous diseases that are associated with dysfunctional inflammation, including chronic wounds.

MATERIALS AND METHODS

Mice

Heterozygous ECRG4 KO mice were purchased from the Mutant Mouse Regional Resource Center supported by the National Institutes of Health and distributed by the University of California, Davis. Briefly, RIKEN complementary DNA 1500015O10 gene coding exon 1 was targeted by homologous recombination in B6/129S5 mixed-background mice. Heterozygous ECRG4 KO mice were then backcrossed to C57/BL6 mice (the Jackson Laboratory, no. 000664) for five generations and subsequently interbred to generate homozygous ECRG4 KO mice on the C57/BL6 background. Ten- to 12-week-old littermate mice were used for experiments involving ECRG4 KOs, and 10- to 12-week-old C57/BL6 mice purchased from the Jackson Laboratory were used as WT mice in experiments without ECRG4 KO mice. The University of California, San Diego (UCSD) Institutional Animal Care and Use Committee approved all animal procedures.

Excisional wound assays

Excisional wound studies were modified from the protocol of Galiano *et al.* (43). Briefly, mice were anesthetized, and dorsal hair was clipped and depilated with Nair (Church & Dwight). Full-thickness excisional skin wounds were created with a 4-mm biopsy punch. A donut-shaped splint with an outer diameter of 10 mm and an inner

diameter of 6 mm was prepared from a 0.5-mm-thick silicone sheet (Grace Bio-Labs, Bend, OR) and sutured on top of the wound using 4-0 nylon monofilament sterile suture (CP Medical, no. CP-B662B-05). Digital photography was used to image each wound daily with a ruler included for scale. Wound area was assessed by planimetry using the National Institutes of Health ImageJ software. Wounds were excised at various time points in some experiments and either fixed in 10% buffered formalin for histological analysis or digested with a Whole Skin Dissociation Kit (Miltenyi Biotec, no. 130-101-540) per manufacturer's instructions to isolate cells for flow cytometry. All experiments were repeated three times, unless otherwise stated, with data pooled and total *n* noted in the figure legends of each experiment.

PVA sponge aseptic wound assay

ECRG4 KO mice and WT littermates, or C57/BL6 mice, were used for PVA sponge implantation assays (28). PVA sponges (7 mm by 3 mm; PVA Unlimited Inc.) were sterilized and hydrated with saline. Anesthetized mice were shaved, an incision was made on the back, and sponges were implanted subcutaneously (28, 29). Incisions were closed with a single 4-0 monofilament nylon suture (CP Medical, no. CP-B662B-05). In one set of assays, 2 μ g of sterile synthetic CT16 peptide (Phoenix Pharmaceuticals, no. 012-24) in phosphate-buffered saline (PBS), or vehicle control, was instilled into the PVA sponges before implantation. Infiltrates were isolated from the sponge in PBS and analyzed by flow cytometry at indicated time points. All experiments were repeated three times, unless otherwise stated, with data pooled and total *n* noted in the figure legends of each experiment.

Flow cytometry

Live cells were collected from mouse bone marrow, blood, spleen, PVA sponges, or digests of mouse wounds and skin, as described above. Cells were incubated with a cocktail of primary antibodies that included specified antibodies: anti-CD11b-APC (allophycocyanin)-Cy7 (BD Pharmingen, 561039; 1:200) and anti-Ly6C-PE (phycoerythrin) (BD, eBioscience, 12-5932-80; 1:200) or anti-CD45-VioGreen (Miltenyi Biotec, 130-110-803), anti-CD11b-APC-Vio770 (Miltenyi Biotec, 130-109-288), anti-Ly6C-APC (Miltenyi Biotec, 130-102-341), anti-CD44-PE-Vio770 (Miltenyi Biotec, 130-110-085), and anti-Ly6G-FITC (fluorescein isothiocyanate) (Miltenyi Biotec, 130-107-912) or Lineage-Biotin cocktail (Miltenyi Biotec, 130-092-613) + anti-biotin-FITC (Miltenyi Biotec, 130-113-852), anti-CD48-VioBlue (Miltenyi Biotec, 130-102-447), anti-Sca1-PE (Miltenyi Biotec, 130-116-489), anti-CD150-PE-Vio770 (SLAM) (Miltenyi Biotec, 130-104-682), and anti-CD117-APC (Miltenyi Biotec, 130-102-492). Propidium iodide (Miltenyi Biotec, 130-093-233) exclusion was used to determine viable cells. HL60 cells were stained with anti-CD44-APC (Miltenyi Biotec, 130-113-338). Flow cytometric and data analyses were performed on a BD Accuri or Miltenyi Biotec MACSQuant10 Flow Cytometer. Data were analyzed with FlowJo software (FlowJo LLC), and statistical analysis was performed as below. All experiments were repeated three times, unless otherwise stated.

Histological analysis

To measure infiltration of Gr1⁺ cells into the wound, tissues were harvested 1 day after injury and frozen in optimal cutting temperature compound. Standard immunohistochemistry was performed

on frozen sections using biotinylated anti-Gr-1 (BD Pharmingen, clone RB6-8C5, no. 553125; 1:200) as the primary antibody and horseradish peroxidase-conjugated streptavidin (Jackson ImmunoResearch, no. 016-030-084; 1:500) as the secondary antibody. Nuclei were counterstained by Mayer's hematoxylin, and slides that were incubated with 1% bovine serum albumin instead of primary antibody were used as negative controls. Immunostaining of tissue sections was imaged with an FSX100 microscope (Olympus). Identification of neutrophils was confirmed by cell and nuclear morphology. Paraffin embedding, sectioning, and hematoxylin and eosin (H&E) stains were performed by the UCSD Tissue Technology Shared Resource Histology Core.

MRSA killing in whole blood

The MRSA strain USA300 LAC was grown on Baird-Parker agar for selection, with individual colonies inoculated in tryptic soy broth and grown to mid-log phase. Bacteria were washed in sterile PBS. One hundred microliters of whole blood from ECRG4 KO or WT mice was inoculated with 10^5 colony-forming units (CFUs) (starting concentration of 1×10^6 CFU/ml), and samples were incubated at 37°C for 3 hours. Serial dilutions of the samples and initial inoculum were prepared in PBS and plated on Baird-Parker agar for enumeration. $n = 4$ mice per group with three technical replicates per mouse.

Cell culture

The acute promyelocytic leukemia promyeloblast human cell line HL60 (ATCC CCL-240) was purchased from the American Type Culture Collection (ATCC) and maintained as recommended in Iscove's modified Dulbecco's medium with 20% fetal bovine serum in an atmosphere of air (95%) and carbon dioxide (5%) at 37°C with replacement of medium and passaging at a cell density between 1×10^5 and 1×10^6 viable cells/ml every 2 to 3 days.

ECRG4 plasmids for stable expression

The full-length human ECRG4 open reading frame was amplified by PCR of a pCMV6-XL4-c2orf40 plasmid (OriGene, no. SC104814) encoding the human ECRG4 transcript (NM_032411) using primers 5'-AGTCCTCGAGCCCCGCCGCGCATGGCTG-3' (forward) and 5'-ATTGGATCCATGGTTAGTAGTCATCGTA-3' (reverse) and cloned into the Xho I and Bam HI cloning sites of pLVX-IRES-ZsGreen1 vector (Clontech, no. 632187) to generate a bicistronic expression vector (lv-ECRG4) that encodes and expresses ECRG4 and green fluorescent protein (GFP). Sequencing confirmed identity of the final plasmid, and the pLVX-IRES-ZsGreen1 plasmid without ECRG4 was used to prepare control GFP⁺ cells (lv-vector). Lentivirus was prepared using a lentivirus packaging system (Clontech) in Lenti-X 293 cells as described by the manufacturer. Viral titers obtained ranged from 2.3×10^7 to 4.0×10^7 used within 3 days on target cells.

Stable expression of ECRG4

HL60 cells were transfected using empty (lv-vector) or ECRG4 (lv-ECRG4) lentivirus using RetroNectin reagent (Takara Inc.) as described by the manufacturer. Five days later, GFP⁺ cells were sorted at the cell sorting facility of the Moores Cancer Center at UCSD and expanded. Two weeks later, GFP⁺ cells were selected a second time by cell sorting, expanded, and passaged, and aliquots of both sorts were frozen in serum-dimethyl sulfoxide.

RNA-seq of gene expression in HL60 cells

In two experiments, vector and ECRG4-expressing HL60 cells were cultured in 35-mm, six-well culture dishes at a concentration of 50,000 cells per well, and RNA was isolated, digested free of DNA, and submitted to genomics core laboratories of Cedars-Sinai (Los Angeles) for RNA-seq analyses. Bioinformatics analyses were performed from FASTQ files, and FPKM (fragments per kilobase of exon model per million reads mapped) was determined by AccuraScience (Iowa), by the UCSD genomics and Cedars-Sinai core, or with public domain resources including STRING (<https://string-db.org>), Cytoscape (<https://cytoscape.org>), and the iDEP.85 web-based tool for RNA-seq data analysis and visualization (<http://bioinformatics.sdstate.edu/idep/>) as indicated.

Quantitative reverse transcription PCR for CD44

RNA was isolated from HL60 cells using an RNeasy mini kit with deoxyribonuclease treatment (Qiagen, no. 74704) according to the manufacturer's recommended procedures, and yields and purity were assessed using a NanoDrop2000 Spectrophotometer (Thermo Fisher Scientific). Complementary DNA synthesis was accomplished with 1 µg of total RNA using iScript (Bio-Rad #170-8891) according to the manufacturer's procedures. CD44 gene expression was analyzed with CD44 primers purchased from OriGene (no. HP200577), and data were normalized to glyceraldehyde-3-phosphate dehydrogenase using forward (CATGAGAAGTATGACAACAGCCT) and reverse (AGTCCTTCCACGATACCAAAAGT) primers purchased from Life Technologies.

Statistical analysis

All statistical analyses were performed using Mstat software (McArdle Laboratory for Cancer Research, University of Wisconsin) or GraphPad Prism (GraphPad Prism Software Inc.). Unpaired two-sample *t* test, Wilcoxon rank sum test, or two-way analysis of variance (ANOVA) with Bonferroni posttest were used. $P < 0.05$ was considered statistically significant. Data are presented as means \pm SD of the mean. Sample size calculations were performed with $\alpha = 0.05$ and $\beta = 0.2$. First derivative of the wound closure plot in Fig. 1A was performed with Prism with default 4 neighbor smoothing.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/6/11/eaay0518/DC1>

Fig. S1. ECRG4 regulates the early response to injury.

Fig. S2. HA signaling pathway from KEGG pathway analysis of RNA-seq data.

Fig. S3. Flow cytometric validation of RNA-seq targets relevant to inflammation.

Fig. S4. ECRG4 KO mice have increased CD44 expression on CD45⁺ leukocytes in blood.

Table S1. Gene Ontology and KEGG pathway visualization of RNA-seq data.

Data file S1. RNA-seq data.

[View/request a protocol for this paper from Bio-protocol.](#)

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